THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 29, Issue of July 18, pp. 18404–18410, 1997
Printed in U.S.A.

Critical Role of Glutamate in a Central Leucine-rich Repeat of Decorin for Interaction with Type I Collagen*

(Received for publication, March 21, 1997, and in revised form, April 29, 1997)

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The chondroitin/dermatan sulfate proteoglycan decorin is known to interact via its core protein with fibrillar collagens, thereby influencing the kinetics of fibril formation and the final diameter of the fibrils. To define the binding site(s) for type I collagen along the core protein, which is mainly composed of leucine-rich repeat structures, decorin cDNAs were constructed and expressed in human kidney 293 cells. The constructs encoded (i) C-terminally truncated molecules, (ii) core proteins with deletions of selected leucine-rich repeats, or (iii) various point mutations. The deletion of the sixth leucine-rich repeat Met^{176}–Lys^{201} and the mutation E180K drastically interfered with the binding to reconstituted type I collagen fibrils. In contrast, the deletion of the seventh repeat Leu^{202}–Ser^{222} led at the most to a marginally impaired binding, although the secretion of this proteoglycan was abnormally low. Decorin with two other point mutations in the sixth leucine-rich repeat, $Lys^{187} \rightarrow Gln$ and $Lys^{200} \rightarrow Gln$, respectively, bound type I collagen either normally or even better than the normal recombinant proteoglycan. These data suggest that a major collagen-binding site of decorin is located within the sixth leucine-rich repeat and that glutamate-180 within this repeat is of special importance for ionic interactions between the two matrix components.

Decorin, a small dermatan sulfate proteoglycan, is a ubiquitous component of extracellular matrices (see Refs. 1 and 2 for reviews) and is synthesized by the majority of cells of mesenchymal origin. It is found preferentially in association with collagen fibrils (3–7), but it can interact with (i) a variety of other extracellular matrix components such as fibronectin (8, 9) and thrombospondin (10), (ii) the transforming growth factor-β family (11), and (iii) a receptor required for its endocytosis (12). It had also been shown that independently of its complex formation with transforming growth factor-β it counteracts the malignant phenotype at least of colon carcinoma cells (13) and suppresses the growth even of normal cells by up-regulating p21 (14).

Most studies on the interaction of decorin with extracellular matrix components concern the binding to type I collagen, although other collagens like type II, III, and VI as well as complement C1q have also been shown to interact with decorin (15, 16). Evidence has been provided that triple helical type I collagen possesses a specific decorin core protein binding site at the d-band in each D-period (4, 5, 17) and that some additional interactions may be mediated by the dermatan sulfate chain (18). As decorin binds to the surface of collagen fibrils, the lateral assembly of individual triple helical collagen molecules is delayed (19, 20), and the diameter of the fibrils is decreased (21). That these interactions are also important *in vivo* can unambiguously be deduced from the phenotype of mice lacking the decorin gene (22). These animals are characterized by fragile skin, and their collagen fibrils have an uneven diameter due to uncontrolled lateral fusion.

There is direct and indirect evidence that collagen-bound decorin is still able to interact with transforming growth factor-β (23), and decorin may even mediate an attachment of type VI collagen to banded collagen fibrils (24). In light of the multitude of potential binding partners of decorin, it would therefore be useful to identify the binding sites for these molecules along the core protein. This would allow an understanding of the assembly of decorin-containing extracellular matrices and of the consequences of a partial proteolytic breakdown of decorin as it happens, for example, in rheumatoid arthritis (25). Decorin belongs to a widely distributed family of proteins that are characterized by about 12 consecutively arranged leucine-rich repeat structures (26, 27), which together form a short β-strand followed by an α-helix. The tertiary structure of one of these proteins has been elucidated by x-ray crystallography at 2.5 Å resolution. The essential features of the molecule are a horseshoe-like structure where the β-sheets form the inner concave surface and the α-helices make up the outer convex face (28). X-ray studies of the complex of ribonuclease inhibitor and its ligand, ribonuclease, indicated that the ligand was in contact with opposing sites of the horseshoe (29) and gave evidence for the conformational flexibility of the leucine-rich repeat structure.

Recently, the re-evaluation of rotary shadowing electron micrographs of a mixture of decorin and of a further proteoglycan made up of leucine-rich repeats, fibromodulin, suggested that decorin, too, is horseshoe-shaped (30). From the results of molecular modeling, it was concluded that the inner concave surface of decorin is of suitable size to accommodate a single triple helix. The possibility of a secondary site located near the C terminus was also considered (31). Studies performed before on chimeric decorin/biglycan proteins had indicated that a major binding site for type I collagen is located within the two repeats Leu^{152}–Lys^{201} (32). Similar conclusions were drawn from own studies employing recombinant decorin peptides (33). The data also suggested the presence of a second, less active binding site in the C-terminal half of the core protein. Similarly, in a preliminary report it was concluded that decorin contains at least two functional domains that are involved in the interaction with the collagen-like molecule C1q (34). On theoretical

* This work was supported in part by the Deutsche Forschungsgemeinschaft (SFB 310, Project B2). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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grounds Scott (30) proposed the sequences Lys^{130}–Arg^{133} and Arg^{272}–His^{275} as binding sites for collagen.

In the present study we describe the type I collagen binding properties of several mutant decorin molecules expressed in and secreted by eukaryotic cells. The results indicate the importance of Met^{176}–Lys^{201}, *i.e.* of the sixth leucine-rich repeat, for collagen binding and indicate a critical role of glutamate 180.

EXPERIMENTAL PROCEDURES

Expression of Recombinant Decorin in Human 293 Kidney Cells—Different authors use nonidentical rules for the numbering of individual amino acids and of leucine-rich repeats of decorin core protein. In this communication we number the amino acids from the start methionine and not from the N terminus of the mature decorin core protein, *i.e.* of the protein obtained after removal of pre- and propeptides. The first leucine-rich repeat is the one comprising Leu^{62}–Asp^{82}, although it can be disputed whether this sequence should be considered as a repeat structure or not.

Clone D6, which contains the complete coding region of human decorin core protein, has been described previously (35). An *Eco*RI/*Hpa*I fragment of this clone was ligated into the pUC18 vector (U. S. Biochemical Corp.) from which subsequently an *Eco*RI/*Xba*I fragment was cloned into the pcDNA3 vector (Invitrogen). The resulting plasmid was used for the expression of recombinant decorin and for the construction of mutated decorin cDNAs. The cDNA for a C-terminally truncated decorin, decorin Asp^{31}–Val^{260} (deletion Asp^{261}–Lys^{359}), was generated as follows. Base pairs 457–898 of a *Hin*cII digest of the cDNA were cloned into the *Bam*HI site of pGem-4Z (Promega), thereby creating a leucine and a stop codon 3′ of the Val^{260} codon. Then an *Eco*RI/*Alw*NI fragment of clone D6 was ligated with the *Alw*NI/*Xba*I fragment from pGem-4Z and cloned into pcDNA3 as above. Decorin exhibiting a deletion of the sixth leucine-rich repeat Met^{176}–Lys^{201} was constructed by a two-step polymerase chain reaction procedure. In the first step 5′- and 3′-portions of the desired sequence were generated by using the primer pairs 5′-CGCCAGTGTGCTGGAATTC-3′ (5′-sequence at the multicloning site) and 5′-GCGGATGTAGGAGAGCTGGTTCAGTCCATTGAAA-3′ (reverse of the overlapping sequence of the 3′-end of the fifth and of the 5′-end of the seventh leucine-rich repeat) and 5′-ACTTTCAATGGACTGAACCAGCTCTCCTACATCCGCATTGC-3′ (forward sequence from the 3′-end of the fifth and the 5′-end of the seventh repeat) and 5′-TATAGAATAGGGCCCTCTAGA-3′ (reverse of the sequence at the 3′-end of the multicloning site), respectively. The amplification conditions were denaturation for 1 min at 94 °C and annealing for 1 min at 55 °C, followed by extension for 2 min at 72 °C and termination for 1 min at 94 °C. The amplified products were purified by agarose gel electrophoresis and subjected to a second polymerase chain reaction using the two primers designed from the multicloning site. The cDNA thus obtained could be ligated into the pcDNA3 vector after *Eco*RI/*Xba*I digestion. The construction of a decorin cDNA with a deletion of the seventh leucine-rich repeat, Leu^{202}–Ser^{222}, followed a similar principle. The additional primers were 5′-CTTCTTCATTCCCTGGAAAGC-3′ (reverse and complement of the 3′-end of the sixth repeat) and 5′-CTTTCCAGGGAATGAAGAAGCTTA-CGGAATTACATCTTGATG-3′ (forward sequence from the 3′-end of the sixth and the 5′-end of the eighth repeat). Polymerase chain reaction primers were 5′-CCAGGGAATGCAGAAGCTCTC-3′ (forward) and 5′-GAGAGCTTCTGCATTCCCTGG-3′ (reverse and complement) for K200Q, 5′-CAATCCGCTGCAGAGCTCAGG-3′ (forward) and 5′-CCTGAGCTCTGCAGCGGATTG-3′ (reverse and complement) for K187Q, and 5′-GATTGTCATAAAACTGGGCACC-3′ (forward) and 5′-GGTGCCCAGTTTTATGACAATC-3′ (reverse and complement) for E180K. Sequencing of the constructs verified the presence of the desired deletions and point mutations and the absence of any point mutations in the remaining coding sequence. A schematic presentation of the various constructs is given in Fig. 1.

Cultured 293 cells were transfected either with the unmodified pcDNA3 vector or with this vector containing one of the above mentioned constructs, employing either the calcium phosphate precipitation method (36) or Lipofectin (Life Technologies, Inc.) according to the recommendations of the manufacturer. The cells were selected for neomycine resistance by adding 750 μg/ml G418 (Life Technologies, Inc.). Transient transfection of COS cells was performed either by the DEAE-dextran (Sigma) method (37) or with Lipofectin.

Metabolic Labeling and Proteoglycan Isolation—Unlabeled reference decorin was purified to about 95% purity from the conditioned medium of cultured human skin fibroblasts as described (10). For the preparation of [^{35}S]sulfate-labeled proteoglycans, confluent fibroblasts or nearly confluent 293 cells were incubated for up to 3 days in the presence of 20 μCi/ml [^{35}S]sulfate (carrier-free, Amersham-Buchler, Braunschweig, Germany) using 10 ml/75 cm^2-culture flask of Eagle's minimum essential medium in which $MgSO_4$ had been replaced by $MgCl_2$ and which was supplemented with nonessential amino acids, penicillin, and 4% (v/v) fetal calf serum. A proteoglycan fraction was obtained from the culture medium by ammonium sulfate precipitation followed by chromatography on DEAE-Trisacryl M (Serva, Heidelberg, Germany) exactly as described (10). Prior to use in binding assays the proteoglycan fraction was dialyzed against phosphate-buffered saline (18 mM sodium phosphate, pH 7.4, 150 mM NaCl) (PBS)[1] in dialysis tubing prewashed with 5% (w/v) bovine serum albumin in PBS.

When indicated, decorin was purified directly from appropriate DEAE fractions by immunoprecipitation using a monospecific polyclonal antiserum against human decorin and immobilizing the immune globulins on protein A-Sepharose (Sigma) as described previously (38). The immune complex was solubilized by a 2-h treatment at 4 °C with 7 M urea in 20 mM Tris/HCl, 0.15 M NaCl, and protease inhibitors. The solution was subjected to ion exchange chromatography on DEAE Trisacryl, first in the presence and then in the absence of urea, for a stepwise removal of IgG and urea and for renaturation of the proteoglycan. Decorin-containing fractions were dialyzed against PBS as described above.

For the preparation of [^{35}S]methionine-labeled proteoglycans, medium was changed to methionine-free Waymouth MAB 87/3 medium supplemented with 4% of dialyzed fetal calf serum. After 1 h of preincubation, labeling medium was added (7 ml/75-cm^2 culture flask), which contained 100 μCi of [^{35}S]methionine (specific radioactivity, 1.07 mCi/μmol; Amersham-Buchler), and incubation continued for up to 6 h. In pulse-chase experiments with [^{35}S]sulfate or [^{35}S]methionine, the chase media contained the 10-fold normal quantity of the unlabeled precursor. Proteoglycans were then obtained by immune precipitation as described above and subjected to digestion with chondroitin ABC lyase (Seikagaku Kogyo, Tokyo, Japan) as quoted earlier (12).

Binding to Reconstituted Type I Collagen Fibrils—Acid-soluble type I collagen from calf skin (Sigma) was dissolved in 17 mM acetic acid (3.3 mg/ml), neutralized by adding an equal volume PBS, and incubated for 1 h at 37 °C. The fibrils formed were collected by centrifugation, suspended in 330 μl of 5% (w/v) bovine serum albumin in PBS/mg of collagen, and dispersed by ultrasonication. In binding assays 50-μl portions of this suspension were mixed with various amounts of [^{35}S]sulfate-labeled proteoglycans yielding a final volume of 600 μl of PBS plus 0.1% Triton X-100. When indicated, unlabeled decorin from human skin fibroblasts (5 μg) or the peptide Val^{129}–Leu^{134} was also added. All assays were done in duplicate or triplicate. Blanks without collagen were always analyzed, although in no case did proteoglycans become insoluble during incubation in the absence of collagen. After 5 h at 37 °C under constant mixing, pellet and supernatant were separated by centrifugation (10,000 $\times$ *g* for 10 min). When immunopurified decorin had been employed, the pellet was washed three times with PBS and dissolved in 1% SDS for direct quantification. When a total proteoglycan fraction was used for binding, the proteoglycan collagen complex was first disrupted enzymatically by incubation for 20 min at 45 °C with 5 bovine tendon collagen units of collagenase (Advance Biofactures, Lynbrook, NY) in 30 μl of 25 mM Tris/HCl, pH 7.2, containing 0.15 M NaCl, and 10 mM $CaCl_2$. It had been ascertained by use of [^{35}S]methionine-labeled decorin and polyacrylamide gel electrophoresis that collagenase from this particular supplier does not degrade the core protein of the proteoglycan. Aliquots of the fraction of the unbound material and of the collagenase digest were then subjected to immune precipitation using the polyclonal antiserum against decorin complexed with protein A-Sepharose as described above. Reprecipitation experiments indicated that the immune precipitation was at least 90% complete.

Other Methods—SDS-polyacrylamide gel electrophoresis followed either by fluorography or by Western blotting was performed as described previously (12, 39).

RESULTS

Expression of Recombinant Decorin Species—Various decorin cDNAs were constructed, all of which contained the glycosaminoglycan chain attachment site but encoded for ei-

[1] The abbreviations used are: PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

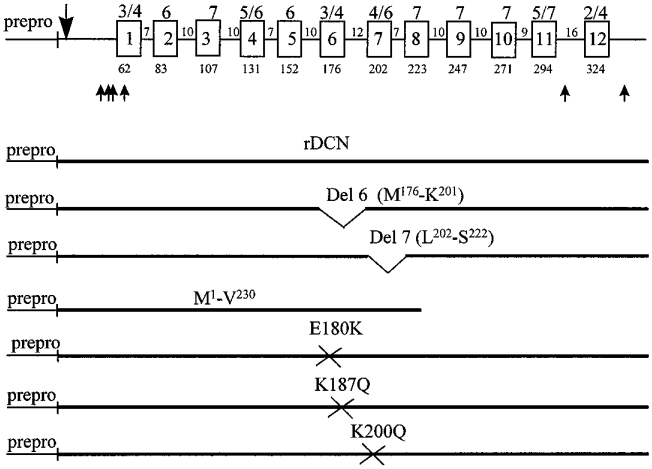

FIG. 1. **Schematic structure of recombinant decorin core protein constructs.** The consensus sequence Leu-Xaa-Xaa-Leu-Xaa-Leu-Xaa-Xaa-Asn-Xaa-(Leu/Ile)-(Ser/Thr)-Xaa-(Val/Ile) is *boxed*. The *numerals* above the *boxes* give the degree of conservation of the seven specified residues without (*first numeral*) or with (*second numeral*) taking into account conservative exchanges. *Small numerals* indicate the number of amino acids between the *boxed* units and the position of the first amino acids of these units, respectively. The glycosaminoglycan attachment site is indicated by a *large arrow*, and cysteine residues are shown by *smaller arrows*. *prepro* indicates the preprosequence that is normally removed intracellularly, and *Del* indicates the deletion of the indicated leucine-rich repeat.

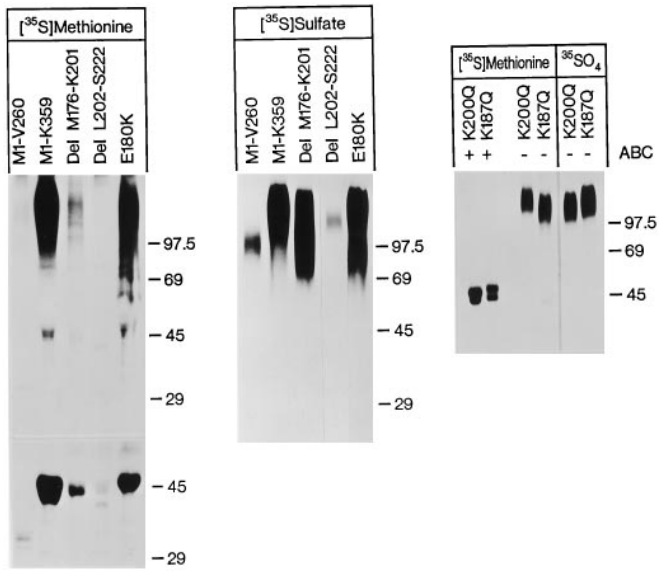

FIG. 2. **Decorin expression by transfected 293 cells.** [^{35}S]Methionine- and [^{35}S]sulfate-labeled decorin was prepared after labeling periods of 6 and 24 h, respectively, by immunoprecipitation of the medium of the cells in a 75-cm^2 flask that had been transfected with the indicated cDNA constructs. The materials were subjected to SDS-PAGE in a 12.5% separation gel. The material obtained after digestion with chondroitin ABC lyase is shown in the *lower part* of the *left* fluorogram and in the lanes marked +*ABC* of the *right* fluorogram. The migration distance of reference protein is indicated on the *right margin*.

ther (i) C-terminally truncated molecules, (ii) deletions of specific leucine-rich repeats, or (iii) point mutations (Fig. 1). All constructs were transiently expressed in COS cells, thereby allowing a quick search for interesting constructs. Unlike NIH3T3 cells, human kidney 293 cells were successfully transfected with all constructs. The isolation of recombinant proteoglycans was facilitated by the availability of monospecific polyclonal antibodies that were reactive toward all decorin constructs under standard immune precipitation conditions. No immunoreactive material was observed in the medium of 293 cells stably transfected with the insert-free pcDNA3 vector, regardless of whether [^{3}H]leucine, [^{35}S]methionine, or [^{35}S]sulfate was used as metabolic precursors (result not shown).

Decorin was labeled with [^{35}S]methionine, immunoprecipitated, digested with chondroitin ABC lyase, electrophoresed on SDS-PAGE, and detected as glycosylated decorin core proteins (Fig. 2). In contrast to normal skin fibroblasts, 293 cells did secrete a small fraction of glycosaminoglycan-free core protein into the culture medium, and this was observed when the chondroitinase ABC lyase treatment was omitted. However, because all further experiments were performed with [^{35}S]sulfate-labeled material purified by anion exchange chromatography, the free core protein was removed and did not complicate the data interpretation. Cells transfected with normal, full-length decorin cDNA secreted a proteoglycan with a core protein that was indistinguishable in its electrophoretic behavior from wild-type decorin expressed in fibroblasts. This suggests that the recombinant core protein is linked similarly to the wild-type protein with two and three asparagine-bound oligosaccharides, leading to the doublet core protein bands seen. Surprisingly, the core protein of decorin E180K did not show the smaller of the two core protein bands, suggesting that all molecules were linked with three *N*-glycans. Deletion of the whole leucine-rich repeat (Del Met^{176}–Lys^{201}) also gave rise to a single core protein band with a somewhat faster mobility than the wild-type core protein carrying three asparagine-bound oligosaccharides. This is suggestive but not proof for the attachment of three *N*-glycosidically linked oligosaccharides. In this context it is interesting to note that decorin K187Q and decorin K200Q yielded two protein bands of somewhat different mobility upon treatment with chondroitin ABC lyase, which could indicate that subtle changes in the sequence of the core protein may influence the processing of the oligosaccharides.

The electrophoretic mobility of [^{35}S]sulfate-labeled proteoglycans is also shown in Fig. 2. Truncation of the core protein or elimination of individual leucine-rich repeats did not result in major differences in the electrophoretic mobility of the broad band of the intact proteoglycan. However, as in the case of [^{35}S]methionine-labeled material, considerable differences were noted between the various decorin constructs in the quantities of [^{35}S]sulfate-labeled proteoglycan secreted into the culture medium. In 293 cells transfected with the full-length decorin cDNA, about 50% of all secreted [^{35}S]sulfate-labeled macromolecules could be precipitated with antibodies against decorin. Similar data were obtained for decorin species carrying point mutations. In five independent experiments, for each preparation of decorin with a deletion of Met^{176}–Lys^{201} and Ley^{202}–Ser^{222}, the percentage or the isotope recovered from the proteoglycan fraction as decorin varied between 25 and 42% and 4 and 10%, respectively. Furthermore, in addition to the lowered proportion, there was also a reduction in the total quantity of incorporated radiosulfate. Truncation of the C-terminally located residues Asp^{231}–Lys^{329} also was accompanied by a lowered proportion of this recombinant proteoglycan in the culture medium (10–15%). This may indicate that some of the mutant proteoglycans could not be transported normally from the endoplasmic reticulum to the plasma membrane. Decreased secretion of decorin carrying deletions of either Met^{176}–Lys^{201} or Leu^{202}–Ser^{222}, however, was not observed in pulse-chase experiments with [^{35}S]sulfate (data not shown). Identical pulse-chase experiments using [^{35}S]methionine could not be performed in 293 cells, because despite the preincubation with methionine-free medium, it took at least 3 h to equilibrate the methionyl tRNA pool with the radioactive amino acid. The data

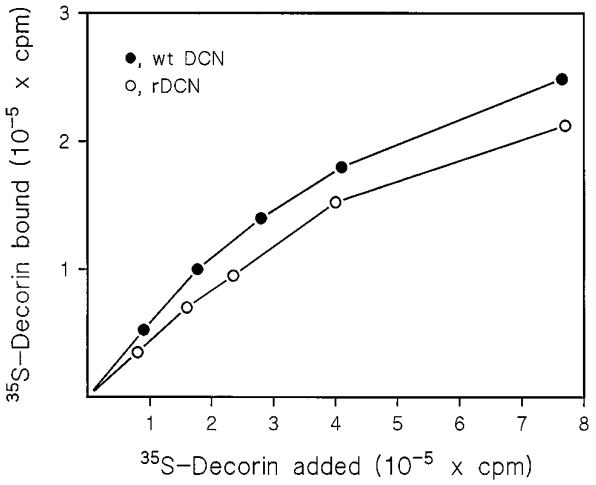

FIG. 3. **Binding of decorin from normal human skin fibroblasts and from transfected 293 cells to reconstituted type I collagen fibrils.** Wild-type decorin (*wt DCN*) from fibroblasts and decorin from 293 cells transfected with the full-length decorin cDNA (*rDCN*) were obtained after a labeling period of 72 h with [^{35}S]sulfate by immunoprecipitation of the conditioned medium.

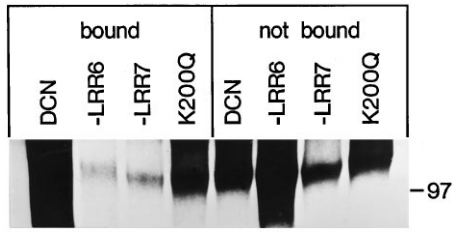

FIG. 4. **Immunoprecipitation of recombinant decorin after collagen binding assays.** Transfected 293 cells were incubated with [^{35}S]sulfate for 24 h. Media were purified on DEAE-Trisacryl columns, and equal aliquots of the proteoglycan fractions were used in collagen binding assays. After collagenase treatment of bound material aliquots representing 50% of the proteoglycan used in the assay were immunoprecipitated with decorin antibodies and subjected to SDS-PAGE in 10% polyacrylamide minigels.

TABLE I

Collagen binding of decorin constructs expressed in 293 cells

[^{35}S]Sulfate-labeled decorin was purified by immune precipitation, and 8,000–12,000 cpm were used for collagen binding. The range of triplicate values is given.

Construct	Total decorin bound
	%
Full-length decorin	66.0–72.1
Deletion Met^{176}–Lys^{201}	16.2–20.4
Deletion Leu^{202}–Ser^{222}	58.3–63.8
Deletion Asp^{261}–Lys^{359}	40.6–47.5
Full-length decorin, E180K	24.1–27.2
Full-length decorin, K187Q	70.1–74.4
Full-length decorin, K200Q	77.2–78.8

obtained after a 3-h pulse, however, indicated that decorin with deletions of leucine-rich repeats was continuously being chased into the medium pool during 19 h, whereas the full-length decorin pulse was completely secreted during the first 4 h of chase. These observations can be explained by the hypothesis that decorin with deletions is transported through the rough endoplasmic reticulum more slowly after protein synthesis, but after it is modified into a proteoglycan in the Golgi network all forms are secreted out of the cell at the same approximate rate. Secreted proteoglycans remained stably in solution and could be reproducibly used for binding studies.

Binding to Reconstituted Type I Collagen Fibrils—Binding of decorin to reconstituted type I collagen fibrils was measured by allowing a crude, native proteoglycan preparation to interact with the fibrils and then quantitating decorin in the unbound and bound fractions. In most cases, the results were verified using immunopurified decorin in the binding assay. The latter method has the disadvantage that the immune complex had to be disrupted by urea and that a renaturation step was required. In the first assay native decorin is used, but other proteoglycans as for example biglycan are also present in the preparation and may compete for binding (40).

In a first set of experiments the binding of wild-type decorin from fibroblast medium and that of recombinant full-length decorin from 293 cells were compared. It is evident from the data of Fig. 3 that both proteoglycan preparations behave very similarly, there being an almost linear relationship between the quantities of added and collagen-bound decorin up to a quantity of about 30,000 cpm/assay. It was assumed, therefore, that a saturation of binding sites for the similarly sized mutated decorin molecules would also not occur at similar doses.

Next, various constructs were labeled with [^{35}S]sulfate in 293 cells, and identical aliquots of a crude proteoglycan preparation from the culture medium were used in a collagen binding assay. Bound and unbound decorin was then isolated by immune precipitation and subjected to SDS-PAGE. The fluorograms are shown in Fig. 4. It is evident that only small quantities of decorin with a deletion of the sixth leucine-rich repeat (Met^{176}–Lys^{201}) were able to interact with type I collagen compared with those deleted in the seventh leucine-rich repeat (Leu^{202}–Ser^{222}).

We had observed previously that about 90% of decorin being retained by a type I collagen affinity column could be desorbed by a NaCl gradient (with 95% of the proteoglycan eluted by 0.5 M NaCl), whereas the solubilization of the remaining 10% required the application of chaotropic reagents.[2] Thus, primarily ionic interactions appear to be responsible for the binding between decorin and type I collagen. For these reasons several charged amino acid residues of the sixth leucine-rich repeat, which are conserved in different species (41), were replaced during site-directed mutagenesis. A comparison of the binding properties of the different decorin constructs is given in Table I. The data corroborate the observation that in contrast to the deletion of Met^{176}–Lys^{201}, the deletion of Leu^{202}–Ser^{222} only slightly affects collagen binding. Truncation of the C terminus (deletion Asp^{261}–Lys^{359}) also lead to an only moderately impaired collagen binding. Interestingly, the replacement of lysine residue 200 by glutamine resulted in an even better collagen binding, a finding that was confirmed in three independent series of experiments. On the other hand, replacement of glutamate 180 by lysine resulted in the production of a proteoglycan whose interaction with type I collagen was strongly reduced, although not to the same extent as upon deletion of the entire leucine-rich repeat structure.

For the most interesting constructs the dose dependence of collagen binding was investigated in greater detail (Fig. 5). It can clearly be seen that decorin with a deletion of Met^{176}–Lys^{201} as well as decorin E180K have an impaired capability for collagen binding at all doses tested. Nonradioactive, wild-type decorin from fibroblasts reduced the binding of all of the radioactive species shown in Fig. 5, as well as all other decorin constructs. Double-reciprocal plots indicated that this inhibition was competitive in nature. However, in agreement with previous findings (33, 32), the relatively high quantity of bound decorin at the lowest doses of decorin exhibiting a deletion of

[2] H. Kresse and J. Rauterberg, unpublished results.

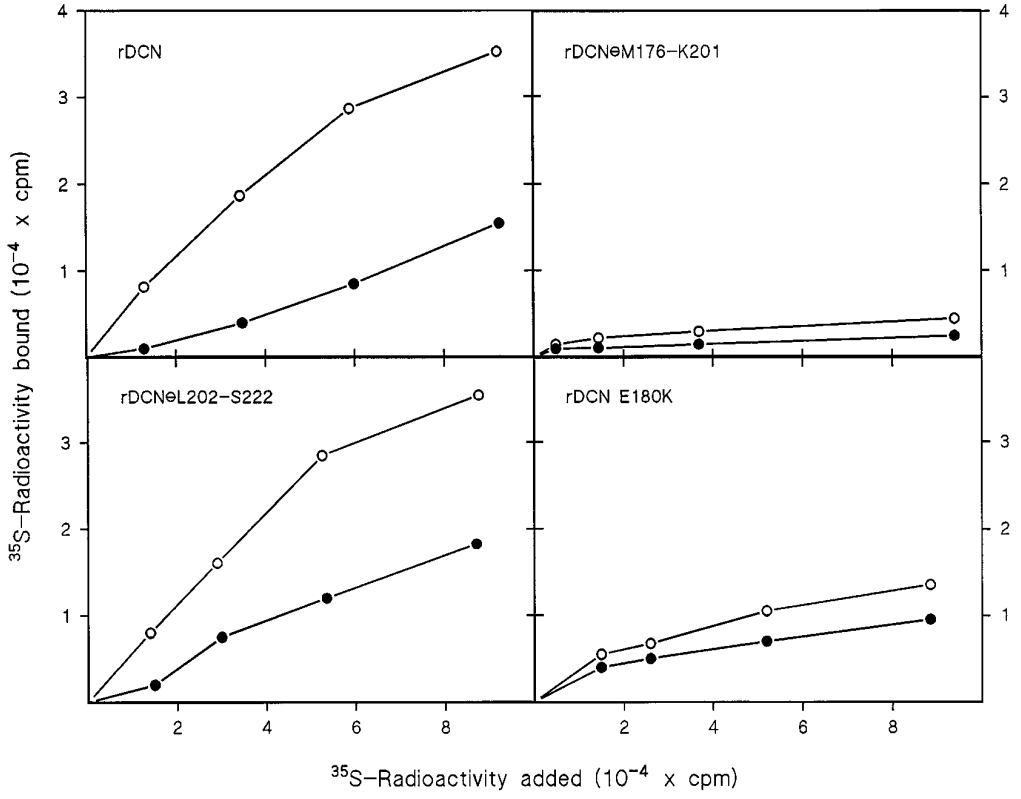

FIG. 5. **Dose dependency of the interaction of recombinant decorin and type I collagen.** [^{35}S]Sulfate-labeled proteoglycans were prepared from transfected 293 cells after a labeling period of 72 h. After purification by anion exchange chromatography, aliquots of the proteoglycan fraction were used for collagen binding in the presence (●) or the absence (○) of 5 μg of unlabeled decorin from fibroblast secretions prior to collagenase digestion and quantification by immunoprecipitation.

Met^{176}–Lys^{201} and of decorin E180K suggested the presence of a second binding site that is not affected in these mutants. From a Scatchard plot (Fig. 6) of these two constructs, it can be concluded that it is specifically the number of binding sites that is reduced in the mutant.

Because the peptide Lys^{130}–Arg^{133} was considered as a binding site for type I collagen (30), peptide Val^{129}–Leu^{134} was tested as competitor of decorin binding. No effect was observed at all concentrations tested (1.5–150 μM) (data not shown).

DISCUSSION

The results of this study provide a further example of the possibility of producing recombinant decorin being *N*-glycosylated and linked with a chondroitin/dermatan sulfate chain (13, 32, 41–45). For the first time, however, deletions of individual leucine-rich repeats, point mutations, and truncation of C-terminal sequences were introduced.

The main result of the present study was the observation that the sixth leucine-rich repeat Met^{176}–Lys^{201} and specifically glutamate 180 within this repeat are of special importance for the interaction with reconstituted type I collagen fibrils. The deletion of just any whole repeat itself is not a necessary condition to interfere with collagen binding because decorin with a deletion of the seventh repeat Leu^{202}–Ser^{222} exhibited, at most, marginally impaired binding properties. Taking into account the horseshoe model of the tertiary structure of decorin and the proposal that collagen triple helices are in contact with the inner concave surface of the proteoglycan (31), it seems likely that there is sufficient flexibility within the core protein to compensate for the loss of at least certain single repeat structures.

Decorin is not the only chondroitin/dermatan sulfate proteoglycan that binds to fibrillar collagens. The homologous proteoglycan, biglycan, has been shown to interact with type I collagen, too, although the dissociation constants obtained from Scatchard plots were higher for glycanated biglycan than for glycanated decorin (40). The sixth leucine-rich repeat of biglycan is homologous with the sixth repeat of decorin, and there is, as in decorin, a glutamate residue at the fifth position of this repeat (46). Thus, the different affinities of decorin and biglycan for type I collagen are unlikely to result from structural differences within this single repeat. It had been observed, however, that glycosaminoglycan-free biglycan exhibited a

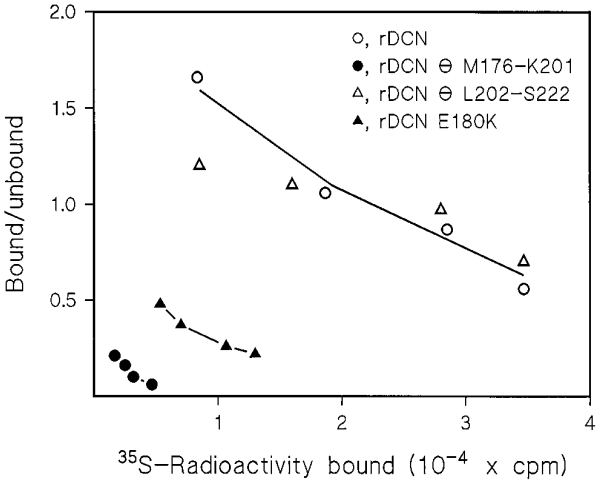

FIG. 6. **Scatchard plot of the interactions of [^{35}S]sulfate-labeled recombinant decorin species with type I collagen fibrils.** The plot was obtained from the data given in Fig. 5.

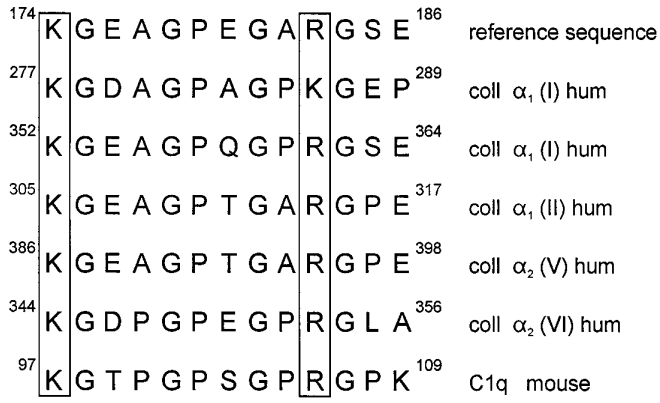

FIG. 7. **Examples of sequences with homology to a putative reference sequence of an α_1 (I) chain at the d-band in the gap zone (31).**

much higher affinity for reconstituted collagen fibrils than the glycanated species (40), and it is interesting to consider that the different affinities of decorin and biglycan for collagen are a reflection of the different number of glycosaminoglycan chains.

Unfortunately, we have not been able to obtain sufficient quantities of pure recombinant proteins under nondenaturing conditions to investigate the tertiary structure by CD spectroscopy. Different CD spectra for decorin purified under denaturing conditions and native decorin had been reported recently (43). In the horseshoe model of decorin the carboxylate group of glutamate 180 is oriented toward the water face at the inner concavity, and it seems likely that also in this case the overall structure of the core protein is stabilized by hydrophobic interactions between the adjacent β-sheets and by interactions between the α-helices at the outer convex surface (31).

The mechanism of collagen fibril assembly in the absence or the presence of decorin is by no means fully understood. It is assumed that decorin inhibits the lateral association of collagen monomers into oligomers, and during the early phases of fibril formation a 1:1 stoichiometry between the two macromolecules may be assumed (19). When collagen fibrils grow laterally this molar ratio changes, and there is less than one proteoglycan per D-period of the fibril (47). Reconstituted collagen fibrils as used in the present study bind maximally one decorin per 20 monomers (48). Thus, it appears that during fibrillogenesis *in vivo* there is a continuous association and dissociation of decorin, whereas in the *in vitro* assays the collagen molecules at the surface of the fibril can stably bind the proteoglycan. Whether or not the binding sites and the binding properties are fully identical during these different stages of interactions remains to be investigated.

The interpretation of the data of binding studies between type I collagen and decorin is further complicated by the proposal made on experimental and theoretical grounds (31, 33, 34, 48) that decorin possesses at least two type I collagen binding sites. Molecular modeling of decorin indicated that its concave face could accommodate a single triple helix. The reference sequence ^{174}KGEAPEGARGSE^{186} has been considered to be present at the contact site between both macromolecules (31) because of the proposal that this sequence is a part of the α_1-chain (I) at the d-band of the D-period (49), *i.e.* at the site of decorin binding. Because decorin also interacts with other fibrillar collagens and the complement component C1q (15, 16, 20, 34, 50), we searched the *Swissprot* data base for sequence homology. Some of the relevant data for N-terminal regions with sequence homology are given in Fig. 7. With respect to the reference sequence only the position of basic amino acid residues was fully conserved. This could indicate that the same site in decorin core protein interacts with the different collagens and C1q, respectively, and it also implicates the importance of acidic residues for collagen binding. From the results of the present study, glutamate 180 should be considered as such a critical residue.

A second collagen binding site has been located in the C-terminal portion of decorin (31, 33). The structural prerequisites for this interaction are not yet known. Studies to address this problem would best be done in dynamic fibrillogenesis assays and may require the creation of additional decorin constructs.

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